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14. ABSTRACT
Trastuzumab, a monoclonal antibody (mAb) targeting HER-2/neu, kills tumor cells by several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC). Strategies that enhance the activity of ADCC effectors, including natural killer (NK) cells, may improve trastuzumab's efficacy. NK cells that encounter trastuzumab-coated, HER2-overexpressing breast cancer cells become activated and express CD137, a costimulatory receptor. CD137 activation, which is dependent on the FcγRIII receptor, occurred both in vitro and in the peripheral blood of women with HER2-expressing breast cancer following trastuzumab treatment. Stimulation of trastuzumab-activated NK cells with an agonistic mAb against CD137 killed breast cancer cells more efficiently in vitro and in multiple HER2+ in vivo models.

15. SUBJECT TERMS
breast cancer, antibody-dependent cell mediated cytotoxicity, natural killer cells, trastuzumab

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INTRODUCTION

Of the 207,000 women diagnosed with breast cancer in the United States in 2010 one quarter have tumors overexpressing the transmembrane receptor tyrosine kinase, HER-2/neu (human epidermal growth factor receptor 2, HER2). These women comprise a disproportionate number of the 40,000 annual breast cancer deaths. Trastuzumab is a humanized monoclonal antibody (mAb) targeting HER2. Despite improving the outcome for this poor prognostic group of patients, response rates in metastatic breast cancer to trastuzumab as monotherapy are limited, approximately 10-15%(1).

Multiple strategies have been investigated to enhance the anti-tumor activity of trastuzumab, which is due, at least in part, to antibody-dependent cell-mediated cytotoxicity (ADCC)(2-5). ADCC is dependent upon immune effector cells, mainly natural killer (NK) cells, binding *via* their Fc receptor (FcγRIII, CD16) to the IgG1 Fc, heavy-chain, portion of trastuzumab(3). This leads to the activation of the NK cells, release of their cytotoxic granules, and lysis of the trastuzumab-bound breast cancer cell(6). Clinical results have shown that patients harboring an FcγRIIIA polymorphism with higher NK affinity for IgG1 have a better response to trastuzumab, further supporting the hypothesis that ADCC, including its mediators, is an important in vivo mechanism of trastuzumab action(7, 8). Additional supporting clinical data demonstrated that responders to neoadjuvant trastuzumab exhibited a four-fold increase in antibody-dependent lytic activity from isolated peripheral blood mononuclear cells compared to that of nonresponders(4). Therefore, augmenting ADCC could increase the clinical efficacy of trastuzumab therapy.

Selectively targeting activated NK cells at the tumor site would be an attractive strategy to improve ADCC without incurring the systemic toxicity of global NK cell stimulation, such as that observed with systemic IL-2 or IL-12(9, 10). Recently, it was shown that human NK cells upon Fc-receptor triggering, such as the interaction with antibody-bound tumor cells, upregulate the inducible costimultory molecule, CD137(11). Once induced to express CD137, we hypothesize that the killing function of these activated NK cells can be enhanced by their exposure to an agonistic mAb against CD137, leading to improved anti-tumor activity. In the current study we investigate this hypothesis that an agonistic mAb against CD137 can enhance the killing of human breast cancer cells by trastuzumab both in vitro and in vivo.

BODY

Human HER2-expressing tumor cells coated with trastuzumab induce the expression of CD137 on human NK cells. Purified NK cells from healthy human subjects were incubated with trastuzumab and breast cancer cell lines (BT474M1, HER18, or SKBR3) expressing HER2. This resulted in robust upregulation of CD137 expression. By contrast incubation of the same human NK cells in the absence of tumor cells, or in the presence of HER2-expressing tumor cells and a non-binding mAb (rituximab) had little effect on CD137 expression (Figure 1A). No induction of CD137 occurred on NK cells following incubation of breast cancer cell lines with trastuzumab in the presence of a breast cancer cell line which does not overexpress HER2 (MCF7) (Figures 1A-B). Similarly, trastuzumab D265A, a trastuzumab variant which does not bind human FcγRs, abrogated the increase in CD137 expression on NK cells following exposure to trastuzumab-coated HER2-expressing tumor cells (Supplementary Figures 1). CD137 upregulation occurred preferentially among CD56^{dim} in comparison to CD56^{hi} NK cells (Figure 1C). The induction of CD137 peaked after 24 hours and was associated with a concurrent decrease in the expression of the FcγRIII (CD16)(Figure 1C). Despite a similar decrease in NK cell expression of CD16 following culture with trastuzumab-bound HER2-expressing tumor cells (Supplementary Figure 2A), NK cells from healthy donors with high affinity polymorphisms of the FcγRIIIA-158 (V/V or F/V) expressed increased levels of CD137 compared to NK cells from donors with a low affinity FcγRIIIA-158 (F/F) polymorphism (Supplementary Figure 2B).

An agonistic anti-CD137 mAb increases trastuzumab-mediated NK cell cytokine secretion and trastuzumab-dependent NK cell-mediated cytotoxicity. NK cells from

healthy donors were co-cultured with trastuzumab-bound HER2-expressing breast cancer cells for 24 hours. Subsequently, the activated NK cells were re-isolated and combined with breast cancer cells under different antibody conditions. The function of these activated NK cells was investigated by measuring secretion of interferon (IFN)-γ and lysis of breast cancer cells. Anti-CD137 agonistic mAb significantly increased trastuzumab-induced IFN-γ secretion when incubated with two of three HER2-overexpressing breast cancer cell lines (SKBR3 and HER18)(Figures 2A-D). Moreover, the anti-CD137 mAb enhanced the ability of these activated NK cells to kill trastuzumab-coated tumor cells (Figures 2E-H). Anti-CD137 mAb alone had minimal effect in the absence of both trastuzumab and HER2-overexpressing breast cancer cells. To confirm the enhanced cytotoxicity, ^{51}Cr labelled-breast cancer cell lines were cultured with NK cells, both un-purified (Supplementary Figure 3) or purified (Figures 3A-D) following activation, in the presence or absence of trastuzumab and anti-CD137 mAb. Again, the combination of anti-CD137 antibodies with trastuzumab induced enhanced ADCC relative to treatment with trastuzumab alone using both un-purified and purified NK cells. This effect was observed only against HER2-overexpressing breast cancer cell lines, confirming that the killing was due to ADCC, even though the NKs had been pre-activated.

To investigate if augmented antibody-mediated cytotoxicity of activated NK cells is restricted to the antibody-coated tumor cell used for NK cell activation, cytotoxicity against HER2-expressing breast cancer as well as CD20$^+$ lymphoma was compared using NK cells activated by trastuzumab-coated breast cancer or rituximab-coated lymphoma. Activated NK cells lysed antibody-coated tumor cells with augmented

cytotoxicity independent of antibody-coated tumor cell used for activation. Anti-CD137 mAb significantly increased trastuzumab-mediated breast cancer cytotoxicity of NK cells activated by culture with either trastuzumab-coated breast cancer or rituximab-coated lymphoma (Supplementary Figures 4A-D). Similar enhancement of rituximab-mediated lymphoma cytotoxicity was observed using NK cells activated by either trastuzumab-coated breast cancer or rituximab-coated lymphoma. Finally, to determine if anti-CD137 mAbs enhance cytotoxicity in the setting of trastuzumab resistance, cytotoxicity against a breast cancer cell line intrinsically resistant to trastuzumab (with maintained expression of HER2, HCC1569) was performed with maintained efficacy observed (Supplementary Figure 5).

Anti-CD137 agonistic mAb enhances anti-breast cancer activity of trastuzumab in vivo. The in vivo ability of anti-CD137 mAbs to enhance trastuzumab's activity was tested in a xenotransplanted human breast cancer (BT474M1 cell line) model in athymic mice. These mice have fully competent NK cells while lacking functional T cells. Anti-CD137 mAb alone had no effect on tumor growth, while trastuzumab had a modest effect (Figures 4A and 4C). We simultaneously explored the effect of trastuzumab and anti-CD137 mAb in various schedules. Since maximal upregulation of CD137 required 24 hours of NK cell exposure to trastuzumab-coated cells in vitro, we aimed to determine the importance of injection sequence. Trastuzumab was injected on day 3 (post-tumor inoculation) and anti-CD137 mAb was injected either on day 2, 3 or 4. Treatment was repeated in all groups 14 days following the first injection. Of the three combination sequences tested, trastuzumab followed by anti-CD137 mAb resulted in the greatest reduction in tumor size and mortality with the opposite order, anti-CD137 mAb followed

by trastuzumab having the least effect both in tumor growth (Figure 4A) and survival (Figure 4B).

Since trastuzumab followed by anti-CD137 antibodies demonstrated more potent antitumor activity in vivo, we chose this injection schedule for subsequent experiments. When the treatment regimen was repeated weekly for three weeks the combination treatment resulted in prolonged control of tumor growth and a significant improvement in survival (Figures 4C-D).

Enhancement of the therapeutic activity of trastuzumab by anti-CD137 mAb is specific to HER2-expressing tumors. To determine if the synergy between anti-HER2 and anti-CD137 mAbs requires specific recognition by the anti-tumor antibody, two tumors differing in HER2 expression were inoculated subcutaneously in individual mice. On the left flank, mice were inoculated with MCF7 cells, which are HER2 low to negative. On the right flank, mice were inoculated with HER18 cells, a transduced derivative of MCF7 cells that stably overexpress HER2 (Figure 5A)(12). Mice then received either trastuzumab monotherapy on day 3 or the combination of trastuzumab on day 3 and anti-CD137 mAb on day 4. This sequential therapy was repeated weekly for three successive weeks. As expected, only the HER2-overexpressing tumors responded to monotherapy with trastuzumab. The addition of anti-CD137 mAb resulted in an enhanced therapeutic effect but only against the HER2-positive tumor that could be targeted by the anti-HER2 mAb (Figures 5B-C).

Anti-CD137 mAb enhances the therapeutic activity of trastuzumab against a human HER2-overexpressing primary breast tumor. Since the combination of trastuzumab and anti-CD137 antibodies demonstrated increased efficacy against HER2-overexpressing cell lines, we next aimed to assess efficacy of the combination therapy using a primary patient xenotransplant model. A primary HER2-overexpressing-breast tumor was successfully engrafted into sub-lethally irradiated SCID mice. After tumors were established the mice were randomized into treatment groups that received either: rat IgG control, trastuzumab, anti-CD137 mAb, or trastuzumab followed 24 hours later by anti-CD137 mAb and repeated weekly for a total of three weeks. The combination of trastuzumab and anti-CD137 mAb was superior to trastuzumab alone, significantly reducing tumor growth and prolonging survival (Figures 5D-E).

Increased expression of CD137 on circulating NK cells in patients receiving trastuzumab therapy. We identified 11 patients with HER2-expressing breast cancer receiving trastuzumab therapy. PBMCs were isolated prior to and following treatment. Time points collected following trastuzumab included 4 hours, 24 hours, and 7 days following completion of infusion (Figure 6A). Reduced expression of CD16^{hi} and increased CD137 expression at short interval time points (24 hours) coincided with a transient loss of circulating NK cells. Seven days following trastuzumab infusion, the percent of PBMCs which are circulating NK cells as well as expression of CD16 and CD137 were similar to baseline. Across the 11 patient cohort, the mean NK cell CD137 expression level at baseline, 4.4%, was similar at 4 hours post-trastuzumab, 4.9%, with a gradual, but significant, increase in CD137 expression at 24 hours, 9.6% (Figure 6B). Grouping all post-trastuzumab samples in comparison to baseline, NK cell CD137

expression increased approximately 2-fold. Interestingly, the greatest magnitude of increase in CD137 expression occurred in 2 patients receiving their first dose of trastuzumab.
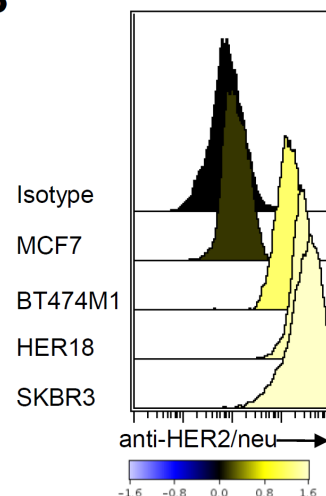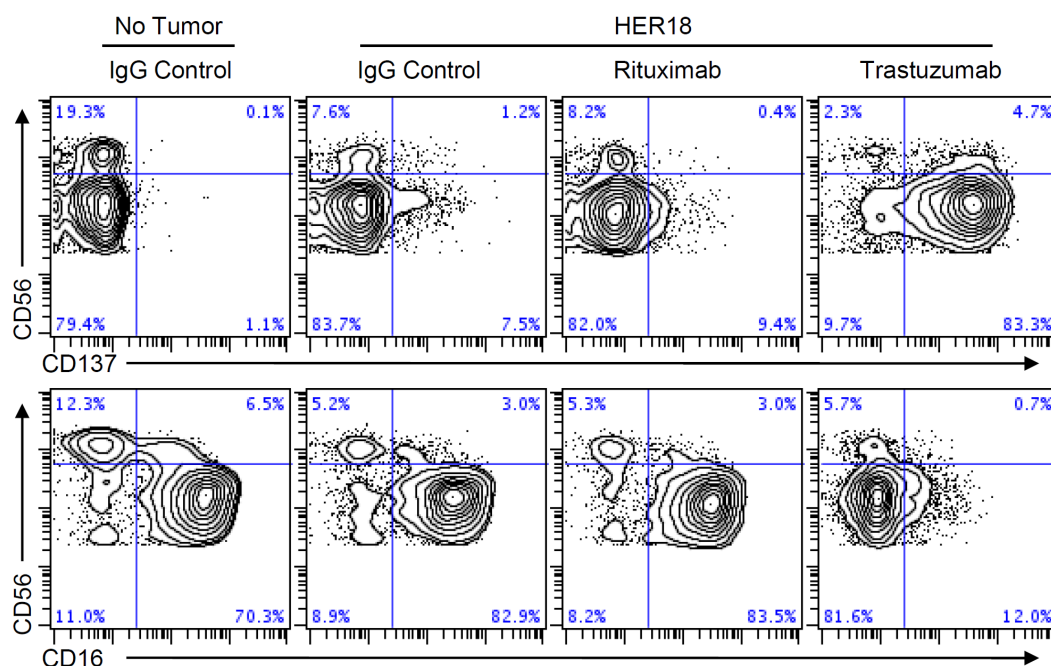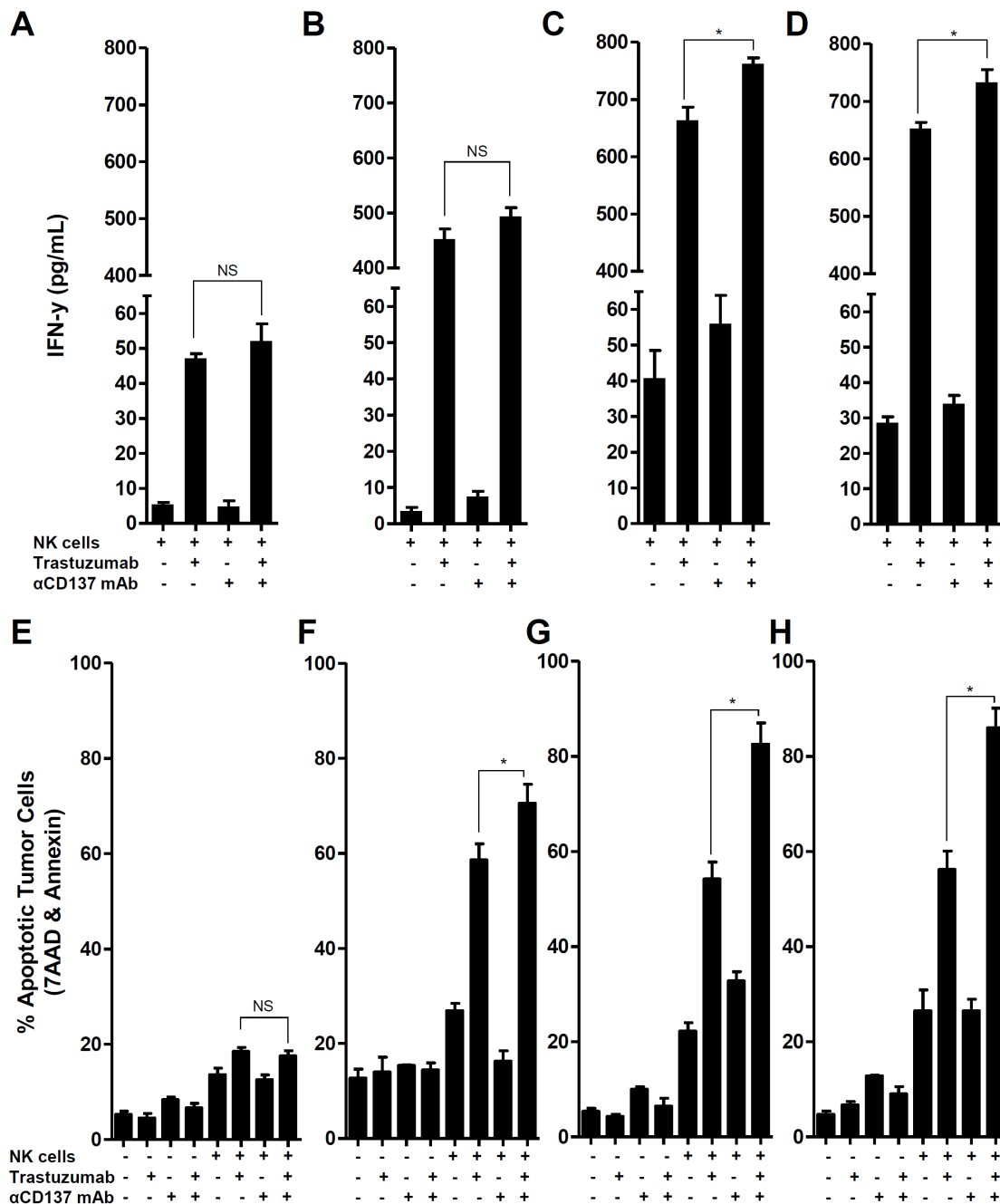
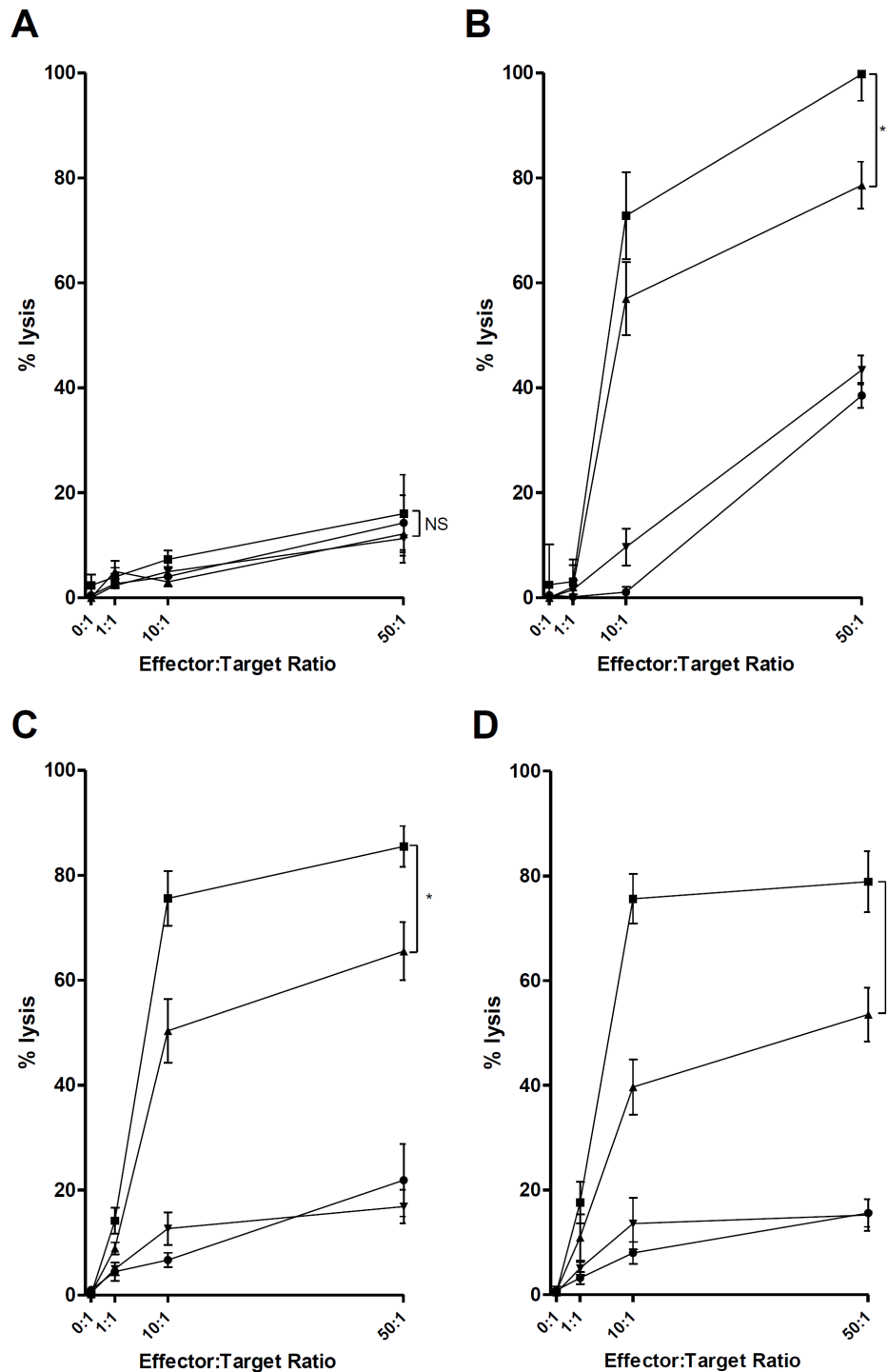**FIGURES**

FIGURE 1

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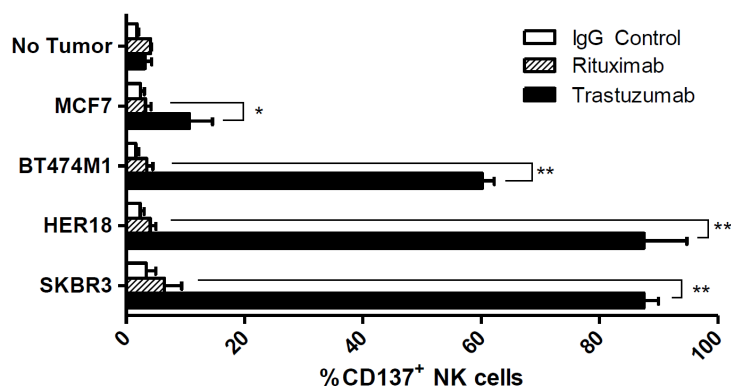
B

C

Figure 1

Trastuzumab induces CD137 upregulation on human NK cells following exposure to HER2-overexpressing tumor cells. Purified NK cells from the peripheral blood of three healthy donors were analyzed for CD137 expression on after 24 hour culture with breast cancer cell lines or no tumor and IgG control, rituximab, or trastuzumab. (A) shows the percentage of CD137$^+$ cells among CD3$^-$CD56$^+$ NK cells from three healthy donors cultured with MCF7, BT474M1, HER18, and SKBR3 breast cancer cell lines (*p=.046, **p<.001). (B) shows HER2 surface expression on breast cancer cell lines with histograms colored according to the log$_{10}$-fold increase in MFI of breast cancer cell line relative to isotype. (C) shows CD137 and CD16 expression on NK cell subsets CD3$^-$CD56^{bight} and CD3$^-$CD56^{dim} from a representative healthy donor after 24 hour culture with IgG control alone, HER18 and IgG control, HER18 and rituximab, or HER18 and trastuzumab.

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Figure 2

Anti-CD137 agonistic mAb increases trastuzumab-mediated NK cell cytokine secretion and cytotoxicity on tumor cells as assayed by cell viability. To evaluate NK cell function, purified NK cells were isolated from three independent, healthy donor PBMCs and cultured for 24 hours together with trastuzumab (10 μg/mL) and irradiated (5,000 rads) breast cancer cells (SKBR3) at a ratio of 1:1. After 24 hours, NK cells were isolated by negative selection and assessed for purity (>90% purity as defined by CD3⁻CD56⁺ flow cytometry) and activation (>50% expression of CD137). Breast cancer cell lines including MCF7 (A and E), BT474M1 (B and F), HER18 (C and G), and SKBR3 (D and H) were cultured for 18 hours with pre-activated, purified NK cells in media alone, or with anti-CD137 mAb (BMS-663513, 10 μg/mL) alone, trastuzumab (10 μg/mL) alone, or trastuzumab plus anti-CD137 mAbs (both at 10 μg/mL) and supernatant was harvested and analyzed by ELISA for interferon-γ (A, MCF7 $p=.39$; B, BT474M1 $p=.16$; C, HER18 *$p=.017$; D, SKBR3 *$p=.034$). Cells were washed and incubated with annexin V and 7-AAD to determine percent apoptotic tumor cells by annexin V and 7-AAD staining (E, MCF7 $p=.43$; F, BT474M1 *$p=.031$; G, HER18 *$p<.001$; H, SKBR3 *$p<.001$).

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Figure 3
Anti-CD137 agonistic mAb increases trastuzumab-mediated NK cell cytotoxicity on tumor cells as assayed by chromium release. To evaluate NK cell cytolytic function, healthy PBMCs were cultured for 24 hours together with trastuzumab (10 µg/mL) and irradiated (5,000 rads) breast cancer cells (SKBR3) at a ratio of 1:1. After 24 hours, NK cells were isolated by negative selection and assessed for purity (>90% purity as defined by $CD3^-CD56^+$ flow cytometry) and activation (>50% expression of CD137). Chromium-labeled breast cancer cell lines including MCF7 (A), BT474M1 (B), HER18 (C), and SKBR3 (D) were cultured for 4 hours with preactivated, purified NK cells in media alone, or with anti-CD137 mAb (BMS-663513, 10 µg/mL) alone, trastuzumab (10 µg/mL) alone, or trastuzumab plus anti-CD137 mAbs. Shown is percent lysis of target cells by chromium release at varying effector (activated NK cells):target cell ratios cultured with media alone(●), anti-CD137(▼), trastuzumab(▲), or trastuzumab and anti-CD137(■) antibodies (A $p=.67$; B *$p=.006$; C *$p=.041$; D *$p=.031$).

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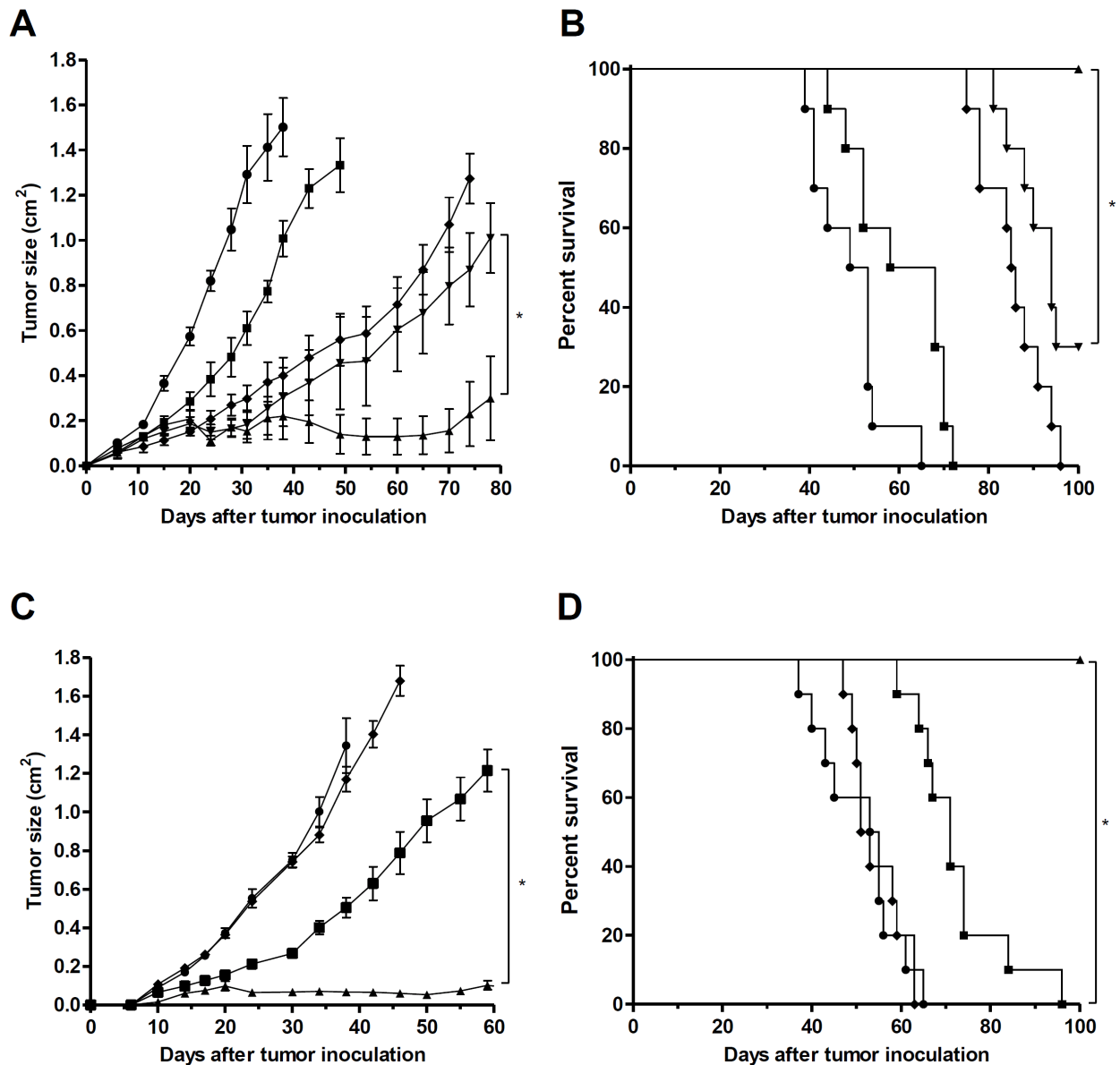
Figure 4

Anti-CD137 agonistic mAb enhances anti-breast cancer activity of trastuzumab in vivo. *Nu/nu* mice were inoculated with $5x10^6$ BT474M1 breast tumor cells, subcutaneously, on the abdomen 1 day after subcutaneous injection of 0.72mg/60 day release beta-estradiol pellet (A-D). (A-B) Post-tumor inoculation, mice then received two cycles of treatment separated by 14 days with either Rat IgG control on days 3 and 17(●),trastuzumab antibody on days 3 and 17(■), trastuzumab antibody on days 3 and 17 and anti-CD137 antibody on days 2 and 16 (♦), trastuzumab antibody on days 3 and 17 and anti-CD137 antibody on days 3 and 17 (▼), or trastuzumab antibody on days 3 and 17 and anti-CD137 antibody on days 4 and 18 (▲). Mice (10 per group) were then monitored for tumor growth (A, *p<.001) and overall survival (B, *p=.001). (C-D) To determine if increased frequency of treatment using the superior combination regimen mice received three weekly treatment with either Rat IgG control starting on day 3(●), trastuzumab antibody starting on day 3(■), anti-CD137 antibody starting on day 4(♦), or trastuzumab antibody starting on day 3 and anti-CD137 antibody starting on day 4 (▲) with treatment repeated weekly for a total of three weeks. Mice (10 per group) were then monitored for tumor growth (C, *p<.001) and overall survival (D, *p=.003).
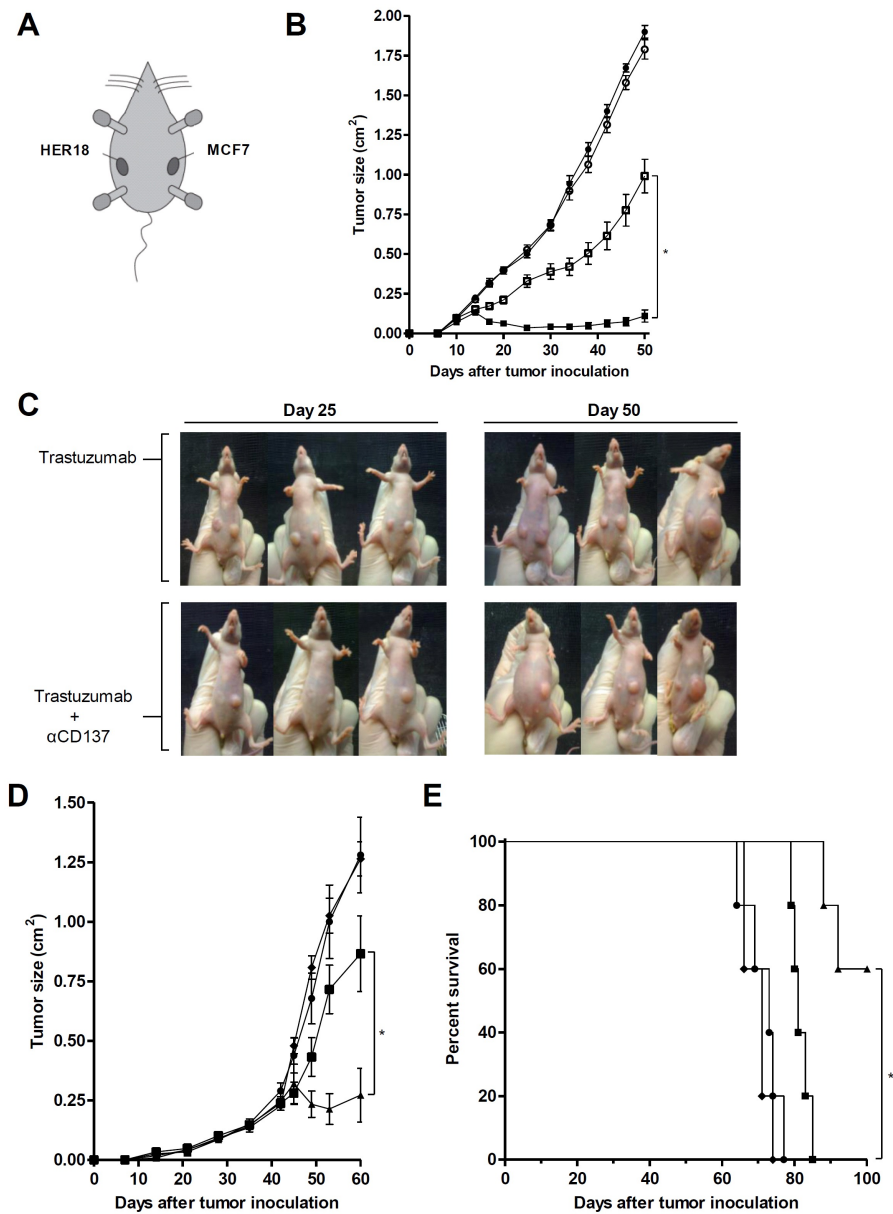
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Figure 5

Anti-CD137 agonistic mAb enhances anti-breast cancer activity of trastuzumab in vivo while retaining HER2 specificity against HER2-overexpressing breast cancer cell lines and a primary breast tumor. *Nu/nu* mice were inoculated with 5×10^6 MCF7 breast tumor cells subcutaneously on the left flank and 5×10^6 HER18 breast tumor cells subcutaneously on the right flank (A, Schema). (A-C) Post-tumor inoculation, mice received either trastuzumab on day 3, or trastuzumab on day 3 and anti-CD137 antibody on day 4 with each treatment repeated weekly for a total of three weeks. (B) Mice (10 per group) were monitored for tumor growth of MCF7 on the left flank (○) and HER18 on the right flank (🍎) in mice treated with trastuzumab, and MCF7 on the left flank (●) and HER18 on the right flank (■) in mice treated with trastuzumab and anti-CD137 mAbs (*p<.001). (C) Representative mice (3 of 10 per group) at 25 and 50 days post-tumor inoculation. SCID mice were inoculated with 1×10^6 HER2$^+$ primary breast tumor cells (SU-258) by intramammary injection 24 hours after 200 cGy total body irradiation (TBI)(D-E). On day 40 mice were randomized to one of four groups (5 mice per group) including IgG control with treatment on day 40 (●), trastuzumab on day 40 (■), anti-CD137 mAb on day 41 (◆), or trastuzumab on day 40 and anti-CD137 mAb on day 41 (▲). Treatment was repeated weekly in each group for a total of three treatments. Mice were monitored for tumor growth (D, *p=.016) and overall survival (E, *p=.002).

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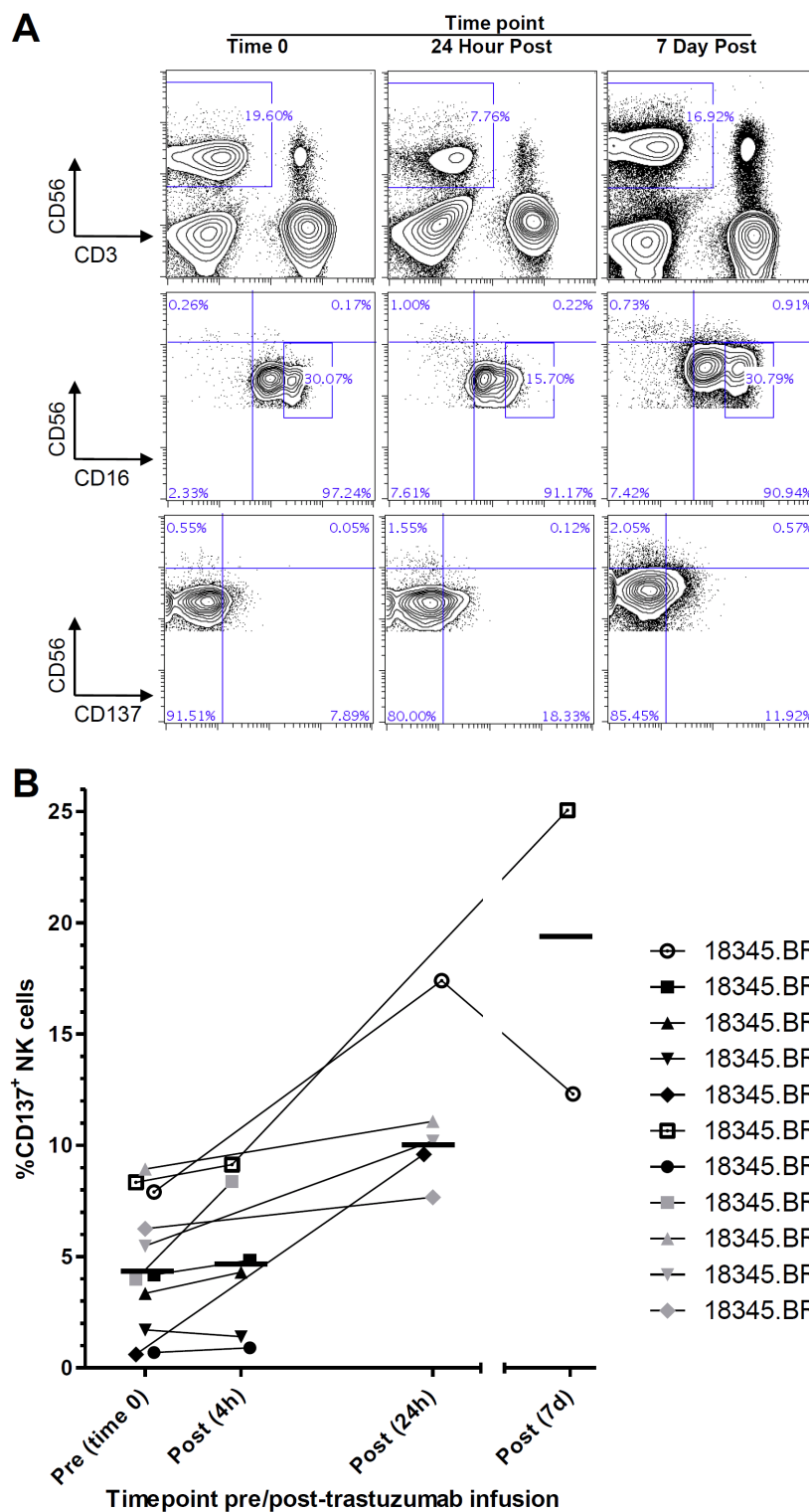
Figure 6

Increased expression of CD137 on circulating NK cells in patients receiving trastuzumab therapy. PBMCs were isolated from 11 patients with HER2-expressing breast cancer prior to and following trastuzumab treatment. (A) shows CD56 and CD3 expression on circulating lymphocytes, and CD16 and CD137 expression on NK cell subsets CD3$^-$CD56^{bight} and CD3$^-$CD56^{dim} from a single representative patient at three time points including prior to trastuzumab, and 24 hours and 7 days following trastuzumab. (B) shows the percentage of CD137$^+$ cells among circulating CD3$^-$CD56$^+$ NK cells from 11 patients prior to and following trastuzumab including two patients receiving their first dose of trastuzumab (patients 18345.BR.001 and 18345.BR.006, open symbols). Mean at each time point denoted by bar.

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KEY RESERCH ACCOMPLISHMENTS

1- Human HER2-expressing tumor cells coated with trastuzumab induce the expression of CD137 on human NK cells.

2- In vitro an agonistic anti-CD137 mAb increases trastuzumab-mediated NK cell cytokine secretion and trastuzumab-dependent NK cell-mediated cytotoxicity.

3- In vivo an agonistic anti-CD137 mAb increases trastuzumab-mediated NK cell cytokine secretion and trastuzumab-dependent NK cell-mediated cytotoxicity.

4- In vivo NK cell expression of CD137 increases following trastuzumab therapy within 24 hours and up to 7 days post, among patients with HER2-expressing breast cancer.

REPORTABLE OUTCOMES

1. **Kohrt, H,** Houot, R, Weiskopf, K, Goldstein, M, Lund, P, Czerwinski, D, Sunwoo, J, Tedder, T, Chen, L, and R Levy. "Stimulation of natural killer cells with an anti-CD137 antibody enhances the efficacy trastuzumab, cetuximab, and rituximab in HER2-expressing breast cancer, EGFR+ head and neck cancer, and CD20+ lymphoma." Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research.

2. **Kohrt, H**, Houot, R, Weiskopf, K, Goldstein, M, Lund, P, Scheeren, F, Czerwinski, D, Colevas, A, Weng, W, Clarke, M, Carlson, R, Sunwoo, J, Tedder, T, Chen, L, and R Levy. "Effect of stimulation of natural killer cells with an anti-CD137 mAb on the efficacy of trastuzumab, cetuximab, and rituximab." Journal of Clinical Oncology, **30**(15_suppl): 2514.

3. Cancer Immunotherapy Consortium, "Stimulation of natural killer cells with an anti-CD137 antibody enhances the efficacy trastuzumab, cetuximab, and rituximab in HER2-expressing breast cancer, EGFR+ head and neck cancer, and CD20+ lymphoma" Annual Meeting, April 26, 2013

4. **Kohrt, H,** Houot, R, Weiskopf, K, Goldstein, M, Scheeren, F, Czerwinski, D, Colevas, AD, Weng, WK, Clarke, M, Carlson, R, Stockdale, F, Mollick, J, Chen, L, and R Levy. "Trastuzumab efficacy in HER2/neu-positive breast cancer is enhanced by stimulation of activated natural killer cells with an antibody to CD137." Journal of Clinical Investigation, **122**(3):1066-1075.

CONCLUSION

Trastuzumab, a monoclonal antibody (mAb) targeting HER-2/neu, kills tumor cells by several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC). Strategies that enhance the activity of ADCC effectors, including natural killer (NK) cells, may improve trastuzumab's efficacy. NK cells that encounter trastuzumab-coated, HER2-overexpressing breast cancer cells become activated and express CD137, a costimulatory receptor. CD137 activation, which is dependent on the FcγRIII receptor, occurred both in vitro and in the peripheral blood of women with HER2-expressing breast cancer following trastuzumab treatment. Stimulation of trastuzumab-activated NK cells with an agonistic mAb against CD137 killed breast cancer cells more efficiently in vitro and in vivo.

One in five women with HER-2/neu-overexpressing breast cancer will relapse despite trastuzumab therapy(13). To address this, multiple strategies have been investigated to enhance trastuzumab's activity and circumvent resistance(14, 15). Strategies currently in various stages of clinical testing have included a small molecule tyrosine kinase inhibitor, generation of bispecific antibodies, antibody-toxin conjugates, dimerization and sheddase inhibitors, and HER2 vaccines(16-20). Here we present an alternative approach aiming at enhancing trastuzumab-mediated ADCC by stimulation of NK cells with an anti-CD137 agonistic mAb. Our results support the following therapeutic strategy. First trastuzumab localizes to the site of the tumor and activates surrounding NK cells *via* Fc-FcγR interaction which increases NK cell expression of CD137. Subsequent administration of an anti-CD137 agonistic mAb stimulates the activated NK

cells to enhance their cytotoxic function while maintaining specificity to lysis of trastuzumab-bound, HER2-overexpressing tumor cells.

CD137 is an inducible, costimulatory molecule expressed on activated CD4 and CD8 T cells, with the majority of prior work focusing on the use of CD137 mAbs or ligands to increase the proliferation and survival of T cells(21). Impressive preclinical activity has been documented with anti-CD137 antibodies in solid tumor models including breast cancer, sarcoma, glioma, colon carcinoma, myeloma, and mastocytoma. These results have motivated clinical trials of agonistic anti-CD137 mAb monotherapy(21-23). CD137 expression, however, is also observed on NK cells, monocytes, dendritic cells, and non-hematopoietic cells where it has only recently have been demonstrated to have functional significance(24, 25). We have previously shown that the anti-lymphoma activity of anti-CD137 agonistic mAb monotherapy requires both CD8 T cells and NK cells(26). Although in the setting of acute myeloid leukemia, expression of CD137 ligand by the leukemic blasts has been shown to impair NK cell spontaneous cytotoxicity(27), additional mouse and human studies have observed enhanced NK cell function including survival and spontaneous cytotoxicity against both hematopoietic and solid tumors(28-31). We recently reported that rituximab-induced ADCC of CD20-expressing lymphoma could be augmented by anti-CD137 agonistic mAbs in multiple mouse models(32).

We show that exposure of human NK cells to trastuzumab-bound, HER2-overexpressing breast cancer results in marked CD137 expression, dependent upon and influenced by the affinity of Fc-FcγR binding. Our findings support prior studies

using immobilized IgG1 or rituximab-coated lymphoma cells to induce CD137 expression(11, 32). Notably, CD137 induction occurs predominantly among the CD56^{dim} subset of NK cells known to mediate ADCC, compared to CD56^{hi} NK cells which are responsible for secretion of cytokines such as IFN-γ(33). We observed a concurrent downregulation of CD16 among the CD56^{dim} subset supporting the internalization of the FcγR following Fc-FcγR binding(34, 35). FcγRIII polymorphisms predicted amplitude of CD137 upregulation, with increased CD137 expression following Fc-FcγR binding of high affinity polymorphisms. In vivo, we expect NK cells in contact with trastuzumab-bound tumor cells to be activated regardless of anatomic site, such as within the primary tumor, tumor-involved lymph nodes, or at distant sites of metastatic breast cancer. In a small series of peripheral blood patient samples obtained prior to and following trastuzumab, downregulation of CD16 and increased expression of CD137 occurred approximately 4 to 24 hours after trastuzumab infusion with marked heterogeneity in magnitude of change. Timing post-trastuzumab infusion, prior exposure to trastuzumab, burden of disease, and genetic polymorphism of FcγRIIIa may contribute to the heterogeneity. As efficacy of anti-CD137 mAb is partially dependent upon expression of CD137 on NK cells, the magnitude and timing of CD137 expression in the peripheral blood or at the tumor site following antibody therapy may provide a predictive biomarker of response.

CD137 stimulation of NK cytotoxicity is restricted to HER2-overexpressing tumors by two mechanisms. First, CD137 upregulation requires contact with trastuzumab-coated tumor cells. For instance, HER-2 negative tumor and/or irrelevant antitumor mAb do not induce upregulation of CD137 on NK cells. Second, CD137 stimulation of activated

(CD137 expressing) NK cells only enhances cytotoxicity towards mAb-coated tumor cells and not uncoated tumor cells. This explains that the enhanced functional activity we observed following in vitro and in vivo stimulation of activated NK cells with anti-CD137 agonistic mAbs maintained specificity to HER2-overexpressing tumors, including in an in vivo, two tumor model. This may translate to a clinical benefit in reduced toxicity of the combination antibody approach as systemic NK cell stimulation should not be expected. Current therapies such as high-dose IL-2, though clinically beneficial, are limited by the systemic toxicity(36, 37). Other approaches such as IL-12 or blockade of inhibitory KIR receptors similarly are unable to selectively target NK cells implicated in trastuzumab-mediated tumor cell killing(36, 38, 39). In the approach developed here, we expect anti-CD137 mAb to preferentially target activated NK cells (i.e. expressing CD137) including NK cells implicated in tumor-directed ADCC while sparing inactive or resting NK cells, thereby limiting potential off-target toxicity of anti-CD137 mAb.

In addition to the dependence on trastuzumab and HER2-overexpressing breast cancer, the combination approach required proper sequence of antibody administration to observe significant enhancement of trastuzumab's activity. In the absence of trastuzumab, we did not observe any level of anti-breast cancer activity of anti-CD137 mAbs either directly or indirectly by stimulation of NK cells in vitro or in vivo. If the murine model recapitulates the human clinical setting, at least in part, then determining this interval between antibodies is critical. Though a 24 hour interval allowed for peak CD137 expression in vitro following NK cell exposure to trastuzumab, based on the first 11 patients assessed prior to and following trastuzumab, the kinetics of CD137 expression are heterogeneous. Accrual of additional patients in the study of CD137

levels may identify the predictive factors. However, if current demographic and clinical-pathologic characteristics do not fully explain the variance, then administration of anti-CD137 mAb could be individually-tailored to the time point of maximal CD137 expression following trastuzumab.

To limit the potential effector cells responsible for breast cancer cell lysis, we used purified NK cells in vitro and, in vivo, partially immunodeficient mouse models. The *nu/nu* and SCID mice are both competent in NK cells, macrophages, and complement but lack a functional adaptive immune response. However, monoclonal antibodies targeting CD137 in vivo likely influence the function of multiple cell types including hematopoietic cells such as granulocytes, T, B, dendritic cells, and monocytes, in addition to NK cells, as well as non-hematopoietic cells(25, 40-43). In particular, our partially immunodeficient mouse models do not explore the impact of trastuzumab and anti-CD137 combination therapy on the adaptive immune response. This may be of importance as we previously observed in an immunocompetent mouse model of lymphoma that anti-CD137 mAbs resulted in the generation of immune memory as illustrated by the protection from re-challenge(44). Recently, Bellati et al. detailed the importance of the adaptive immune response to trastuzumab therapy in its clinical efficacy(45). Taken together, we hypothesize that anti-CD137 agonistic mAb following trastuzumab may also improve adaptive immunity in addition to enhancing ADCC. This hypothesis is supported by recent work by Stagg et al.(46). Recognizing that anti-CD137 mAbs enhance both adaptive and innate immune responses, Fc engineering may further improve efficacy by optimizing the binding to FcRIIB, recently recognized to be necessary for anti-TNFR mAbs' agonistic effects on adaptive immunity.(47)

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Stimulation of CD137-expressing non-NK cells may also induce toxicity including off-target hepatocyte toxicity due to CD8 T cell-dependent hepatitis which has been observed in both mouse models and a phase I clinical trial of anti-CD137 monotherapy(48, 49). However, given the synergy between trastuzumab and anti-CD137 mAb, combination with a tumor-directed mAb may allow the use of minimal doses of anti-CD137 mAb limiting toxicities while retaining anti-tumor activity.

An area worthy of further investigation is the setting of CD137 stimulation in trastuzumab resistance. Clinical studies have been unable to demonstrate decreased HER2 receptor levels or blocked trastuzumab binding capacity among patients with trastuzumab resistant disease(4, 50). Using a cell line with intrinsic resistance to trastuzumab, without loss of surface HER2 expression and due to either expression of t-DARPP(51) or E-cadherin(52), we observed preserved ability of anti-CD137 mAb to enhance trastuzumab-dependent cytotoxicity. Therefore, even in instances of diminished response to trastuzumab, enhancing ADCC with CD137 stimulation may offer the opportunity to overcome tumor resistance.

In conclusion, the results of our study demonstrate that anti-CD137 mAb can enhance trastuzumab's activity by stimulating the immune response to trastuzumab-bound, HER2-overexpressing breast cancer in vitro and in xenotransplant models. This approach is dependent upon sequential targeting of first the tumor by trastuzumab, and second the activated NK cells by anti-CD137 agonistic mAbs. This novel antibody strategy targeting both the tumor and the immune system may thus offer new opportunities to enhance the efficacy of therapeutic antibodies in cancer. This approach

is widely applicable across cancers, including rituximab and CD20-expressing lymphomas(44), cetuximab and EGFR-expressing head and neck as well as colorectal tumors, and yet undeveloped antibodies to currently unknown tumor antigens. Clinical investigations are now warranted to determine if this hypothesis holds true in patients.

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